Interaction between *Streptococcus* spp. and *Veillonella tobetsuensis* in the Early Stages of Oral Biofilm Formation

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Dental plaque is a multispecies oral biofilm, the development of which is initiated by adherence of the pioneer *Streptococcus* spp. Oral *Veillonella* spp., including *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis*, are known as early colonizers in oral biofilm formation. These species have been reported to coaggregate with *Streptococcus* spp. in a metabolic cooperation-dependent manner to form biofilms in human oral cavities, especially in the early stages of biofilm formation. However, in our previous study, *Streptococcus gordonii* showed biofilm formation to the greatest extent in the presence of *V. tobetsuensis*, without coaggregation between species. These results suggest that *V. tobetsuensis* produces signaling molecules that promote the proliferation of *S. gordonii* in biofilm formation. It is well known in many bacterial species that the quorum-sensing (QS) system regulates diverse functions such as biofilm formation. However, little is known about the QS system with autoinducers (AIs) with respect to *Veillonella and Streptococcus* spp. Recently, autoinducer 1 (AI-1) and AI-2 were detected and identified in the culture supernatants of *V. tobetsuensis* as strong signaling molecules in biofilm formation with *S. gordonii*. In particular, the supernatant from *V. tobetsuensis* showed the highest AI-2 activity among 6 oral *Veillonella* species, indicating that AIs, mainly AI-2, produced by *V. tobetsuensis* may be important factors and may facilitate biofilm formation of *S. gordonii*. Clarifying the mechanism that underlies the QS system between *S. gordonii* and *V. tobetsuensis* may lead to the development of novel methods for the prevention of oral infectious diseases caused by oral biofilms.

Bacteria exist as multispecies communities in nature, and signaling among the cells is thought to be a part of the community dynamics. A biofilm is a community of bacteria attached to a substratum or surface. The bacteria in biofilms are embedded in an extracellular polymeric matrix produced by the bacteria themselves. Bacteria develop biofilms on submerged surfaces such as natural aquatic systems, water pipes, living tissues, tooth surfaces, indwelling medical devices, and implants (1). When bacteria succeed in forming a biofilm within a human host, they become highly resistant to antimicrobial treatment (2). Human dental plaque is a well-recognized example of a natural biofilm that plays an important role in the development and pathogenesis of oral diseases such as caries, gingivitis, and periodontitis (3).

The human oral cavity contains more than 19,000 phylotypes of microbial species (4), and approximately 100 to 200 species are found in a single individual (5). Dental plaque is a multispecies biofilm, the development of which is initiated by the adherence of pioneer species to the salivary proteins and glycoproteins adsorbed on tooth enamel. The biofilm is not formed by random simultaneous colonization by these species but rather by selective, reproducible, sequential colonization (6, 7).

The genus *Veillonella* consists of small, strictly anaerobic, Gram-negative cocci that lack flagella, spores, and capsules. Members of the genus *Veillonella* gain energy from the utilization of short-chain organic acids and have been isolated from the oral cavity and intestinal tract of humans and other animals (8, 9). Currently, the genus *Veillonella* is subdivided into 13 species: *V. atypica*, *V. caviae*, *V. criceti*, *V. denticariosi*, *V. dispar*, *V. magna*, *V. montpellierensis*, *V. parvula*, *V. ratti*, *V. rodentium*, *V. rogosae*, *V. seminalis*, and *V. tobetsuensis* (10–17). These *Veillonella* species have been isolated from lesions associated with endocarditis (18–21), hepatic abscesses (22), meningitis (23), osteomyelitis (24), acute pyelonephritis, secondary bacteremia during pregnancy (25), op-

portunistic infections (26–28), and prosthetic joint infection (29). Additionally, these bacteria, like other anaerobes, are susceptible to various antimicrobials; however, several of these species are resistant to tetracycline. In periodontal patients undergoing therapy, *Veillonella* species, along with *Streptococcus* and *Neisseria* species, were found to be consistently resistant to tetracycline (30). Moreover, tetracycline-resistant *Veillonella* species have the opportunity to come in close contact with and, consequently, transfer resistance elements to other oral bacteria and the bacteria that pass through the oral cavity (31). *Veillonella* species were previously known to be sensitive to penicillin and ampicillin but are now frequently resistant to these antibiotics (32).

Of the above-mentioned *Veillonella* species, only *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis* had been isolated previously from human oral cavities as oral *Veillonella* spp. (10, 11, 13, 14, 17). Recently, *V. tobetsuensis* was isolated from a human tongue biofilm and established as a novel species in the genus *Veillonella* in our laboratory (17). The main habitats of these oral *Veillonella* species are the tongue, buccal mucosa, and saliva (9, 10, 33–36). Oral *Veillonella* species, especially *V. parvula*, have been associated with severe early childhood

caries ([37]) and intraradicular infections ([38], [39]), including abscess ([40]), apical root canals ([41]), and dentinal tubules ([42]).

Veillonella species are also predominantly found in subgingival biofilm samples of patients who have chronic periodontitis ([43], [44]). Delwiche et al. ([8]) summarized the implication of *Veillonella* spp. in periodontal diseases as follows: (i) *Veillonella* spp. constitute a part of the microbial community of biofilm and become more prominent as the biofilm develops; (ii) *Veillonella* spp. produce a large amount of lipopolysaccharides (LPS); and (iii) *Veillonella* spp. facilitate associations with other oral microbes that help the *Veillonella* spp. to become established in the oral microbial ecosystem. We further expand on the simplified hypothesis presented above by stating that, given a steady diet of sucrose, the streptococci present can dissimilate it into its component sugars glucose and fructose. Much of the glucose can be converted to lactate, which *Veillonella* spp. can utilize as a carbon and energy source for growth. Some of the sucrose can be converted to dextran by the streptococcal extracellular glucosyltransferase, which is used by *Veillonella* spp. to adhere to teeth and settle ([8]). Free fructose, formed in the process of dextran synthesis by the glucosyltransferase reaction, can be incorporated into the *Veillonella* LPS ([45]) and may be of major significance in the production of LPS.

In the case of dental caries, *Veillonella* species are highly associated with lactic acid-producing species ([46]). This is not surprising given its reliance on lactate as a nutrient source. This has potential clinical utility; *Veillonella* levels may serve as a sensitive biologic indicator and early warning sign of acid production. Among children without history of caries, the presence of *Veillonella* or other acid-producing bacteria, including *Streptococcus mutans*, has predicted the development of future caries ([46]).

As mentioned above, it is evident that oral *Veillonella* species are associated with oral biofilms, which cause many human oral infectious diseases, such as periodontal diseases and dental caries. Therefore, it would appear that understanding the interactions between *Streptococcus* and *Veillonella* spp. in the early stages of oral biofilm formation is important to prevent these oral infectious diseases. However, the detailed roles of oral *Veillonella* species in biofilm formation have not been fully clarified.

In this review article, we summarize the interactions between *Streptococcus* species and *Veillonella* species, especially between *S. gordonii* and *V. tobetsuensis*, in the early stages of oral biofilm formation. Furthermore, the roles of oral *Veillonella* spp. in the early stages of biofilm formation are summarized.

GENERAL RELATIONSHIPS BETWEEN *STREPTOCOCCUS* SPP. AND *VEILLONELLA* SPP. IN ORAL BIOFILMS

Many bacteria rely on metabolic cooperation based on the close proximity of cells for growth and become incorporated within oral microbial communities. *Veillonella* species occur in high abundance in oral biofilms ([47]). Furthermore, they are a part of the pioneer oral communities after birth ([48]). *Veillonella* species, except *V. seminalis*, can utilize short-chain organic acids—especially lactate—for growth. Growth of *Streptococcus* species leads to the formation of lactate, which is a favored substrate of *Veillonella* species. This in turn accelerates the glycolytic rate in *Streptococcus* species by removing the end product (lactate) inhibition. For example, when *Streptococcus gordonii* and *Veillonella atypica* are grown in coculture, a *Veillonella* diffusible signal leads to the upregulation of the *S. gordonii amyB* amylase gene. Increased am-

ylase activity on a starch substrate produces more fermentable glucose, generating further lactate and more favorable conditions for *V. atypica* ([49]).

It has been suggested that the general idea of coaggregation, as well as the subsequent metabolic cooperation, is of major importance for biofilm formation. Currently, there are two major hypotheses that address the coaggregation between *Streptococcus* species and *Veillonella* species.

Organic acids are excreted by *Streptococcus* species during growth on sugars and are the basis for the metabolic communication documented *in vitro* ([50]) and *in vivo* in gnotobiotic rats ([51], [52]). Moreover, it has been shown *in vivo* using rats that *Veillonella* species are not capable of colonizing the tooth surface without *Streptococcus* species as metabolic partners and that larger populations of *Veillonella* species develop in coculture with *Streptococcus* species with which they do not coaggregate ([53]).

On the other hand, Hughes et al. ([54]) stated that the proximity of producer to consumer could be an important factor in facilitating such metabolite transfers; indeed, 83% of oral *Veillonella* species isolated from subgingival plaque were found to be coaggregated with multiple streptococcal reference strains. Moreover, Chalmers et al. ([55]) reported that intrageneric coaggregation of oral *Streptococcus* species and intergeneric coaggregation of oral *Streptococcus* and *Veillonella* species are important factors in the initial formation of spatially distinct and metabolically cooperative communities during primary colonization of the tooth surface. *Streptococcus*-*Veillonella* communities containing coaggregation partners were micromanipulated from human oral biofilm, providing additional evidence of the close association of these two species *in vivo*. In addition, when *Veillonella* species were juxtaposed with coaggregation receptor polysaccharide-bearing *Streptococcus* species in early communities *in vivo*, a rapid succession of *Veillonella* phylotypes was found to occur ([56]). In our present study, there was no coaggregation between *S. gordonii* and *V. tobetsuensis* (data not shown). These results suggested that whether or not coaggregation between *Streptococcus* and *Veillonella* species occurred, the results of such coaggregation would certainly differ from those represented by the combination of the two species in the absence of coaggregation.

The conversion of lactate formed by *Streptococcus* species to less potent acids, such as acetic acid, by *Veillonella* species has been assumed to reduce susceptibility to caries in the host, although little experimental evidence supports this hypothesis. Instead, the results of a molecular study suggest that *Veillonella* species are present together with *Streptococcus* species in caries lesions ([57]).

These reports offer considerable evidence that *Streptococcus* species and *Veillonella* species are linked in oral biofilms.

VEILLONELLA TOBETSUENSIS SP. NOV.

In our previous study, we isolated four unknown *Veillonella*-like strains, which grew on *Veillonella* agar ([58]), from the tongue biofilm of healthy human adults, aged 23 to 26 years. PCR assays with species-specific primer sets designed for the five oral *Veillonella* species, *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, and *V. rogosae*, based on a highly variable region in the *rpoB* gene showed that the four strains were *Veillonella* negative ([59]). However, DNA isolated from these strains generated PCR products with *Veillonella* genus-specific primers ([36]). Subsequently, on the basis of the results of morphological analysis, biochemical analysis, analysis of
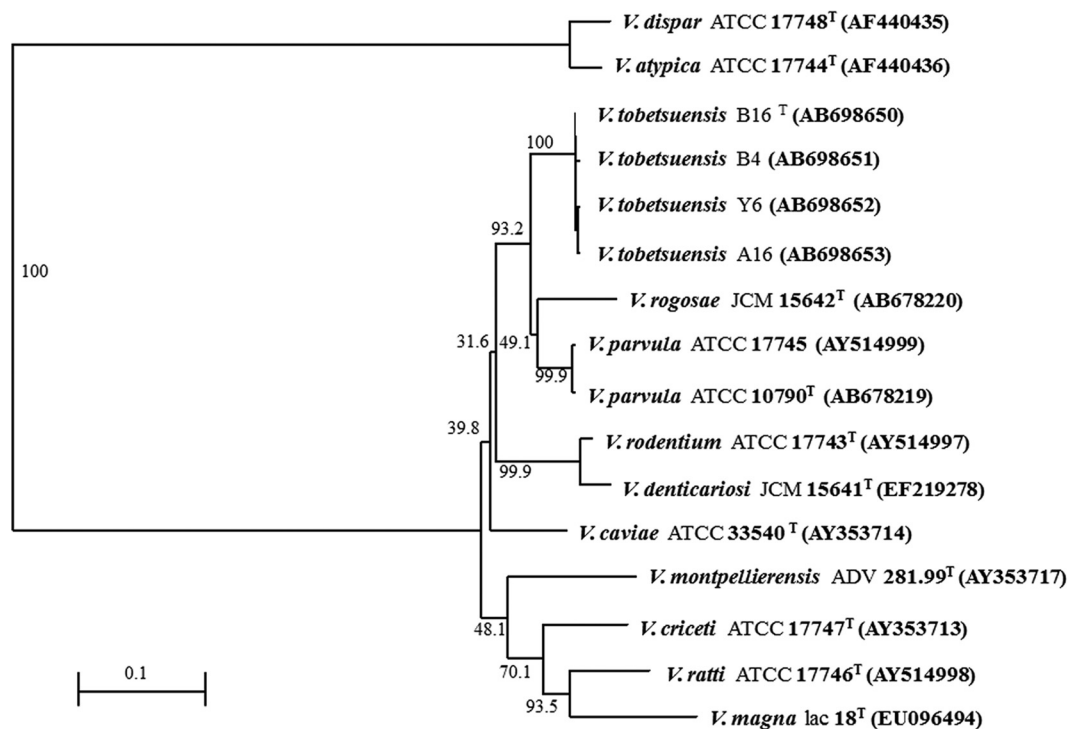
FIG 1 Phylogenetic tree based on *dnaK* sequences.

the composition of cellular fatty acids, and phylogenetic analysis, they were subsequently established as strains of a novel *Veillonella* species, *Veillonella tobetsuensis*.

The type strain of *Veillonella tobetsuensis* is ATCC BAA-2400^T (=JCM 17976^T) isolated from the tongue biofilm of healthy 26-year-old human adults (17). Cells are coccoid (0.3 to 0.7 μm in diameter) and occur singly or in pairs. They are obligate anaerobes, Gram-negative, nonmotile, and nonsporulating, with a convoluted surface. Colonies on brain heart infusion (BHI) blood agar are 0.5 to 2 mm in diameter without a zone of hemolysis and appear as circular, smooth, opaque, and grayish-white colonies after 5 days of incubation under anaerobic conditions at 37°C. The decolorization of basic fuchsin was not observed in the area around the colony in the *Veillonella* agar selective medium. Cells examined under aerobic conditions are all negative for catalase and positive for nitrate reduction. Cells do not produce acids from carbohydrates and do not exhibit extracellular glycosidic enzyme activities. Alkaline phosphatase, pyroglutamic acid arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase are present, and gas is not produced under anaerobic conditions in TGY medium. The major acid end products under anaerobic conditions are acetic acid and propionic acid. The major cellular fatty acids produced are $C_{13:0}$ and $C_{17:1}\omega8$, consistent with other *Veillonella* species. Strains of this species can be differentiated from other *Veillonella* species by *dnaK* and *rpoB* sequence analysis (Fig. 1 and 2) (17).

To determine the distribution and frequency of *V. tobetsuensis*, a species-specific PCR primer pair was previously designed based on the nucleotide sequence of the 70-kDa heat shock protein (*dnaK*) gene of *V. tobetsuensis* ATCC BAA-2400^T. When the tongue biofilm of healthy human adults (22 to 29 years of age) was examined, *V. tobetsuensis* was detected in 5 of 27 subjects and was recovered from 19% (5/27) of the subjects carrying other *Veillonella* species. The prevalence of *V. tobetsuensis* ranged from 7.6% to 20.0% in these subjects (60).

THE BIOFILM FORMED BY *STREPTOCOCCUS* SPECIES AND *V. TOBETSUENSIS*

It has been suggested that oral *Veillonella* species in multispecies communities, especially those that include oral *Streptococcus* species, play a central role in biofilm formation as early colonizers and facilitate the succession of species in developing dental plaque *in vivo* (61).

In our previous study, the influence of each of the 6 oral *Veillonella* species on the formation of biofilms for each of 4 *Streptococcus* species, *Streptococcus gordonii*, *S. mutans*, *S. salivarius*, and *S. sanguinis*, was examined (in 24 combinations) by using a novel method for experimental biofilm formation (62, 63). Biofilm formation was highest in the combination of *S. gordonii* with *V. tobetsuensis*, and biofilm changes with time were particularly noticeable with that combination compared to the other combinations tested. As shown in Fig. 3a, panel II, both the amount of biofilm and the proportion of *V. tobetsuensis* cells in the biofilm increased with time. On the other hand, in the coculture of *S. gordonii* with *V. tobetsuensis*, *V. tobetsuensis* made up a greater proportion of planktonic cells as the number of planktonic cells increased with time (Fig. 3b, panel II). In addition, there was no coaggregation observed between *S. gordonii* and *V. tobetsuensis*, as reported previously (54, 55). Our results further support a study by McBride and van der Hoeven (53), the results of which suggest that there may be specific relationships between *S. gordonii* and *V. tobetsuensis*. For example, *S. gordonii* may provide some factors to promote the growth of *V. tobetsuensis* in the planktonic state
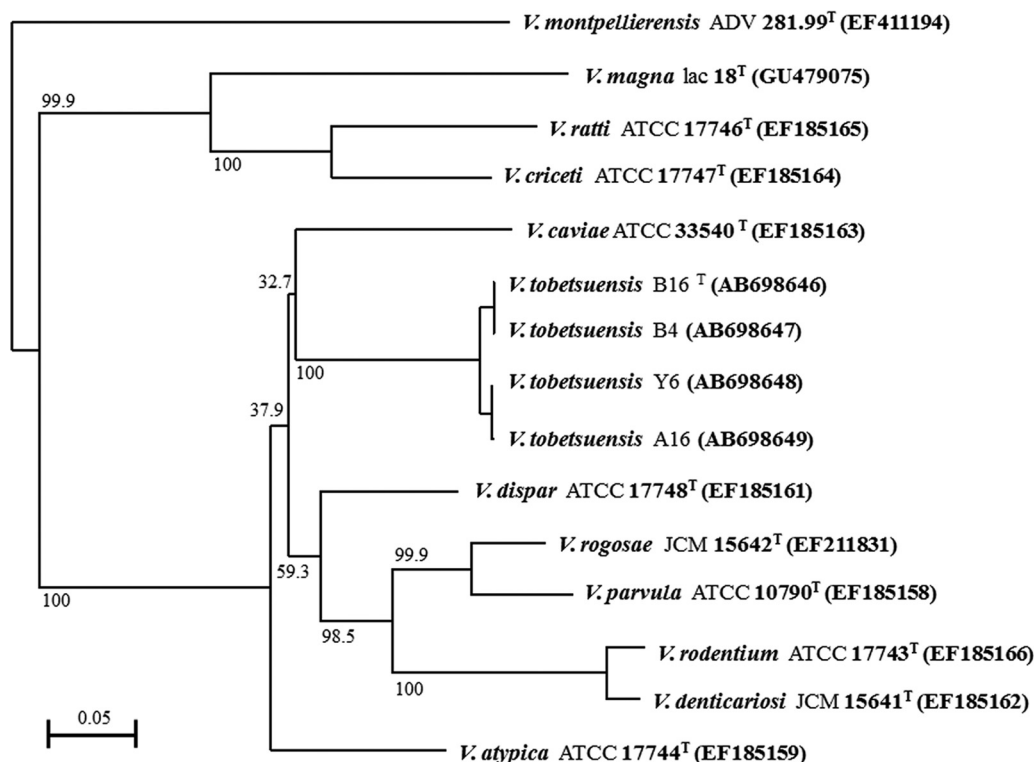
FIG 2 Phylogenetic tree based on *rpoB* sequences.

and/or *V. tobetsuensis* may produce molecules to promote the proliferation of *S. gordonii* in biofilm formation.

QS SYSTEM IN *STREPTOCOCCUS* SPECIES AND *VEILLONELLA* SPECIES

The quorum-sensing (QS) system has been described in both Gram-negative and Gram-positive bacteria. The QS is a bacterial intercommunication system that controls the expression of mul-

tiple genes in response to population density. The basic mechanisms that control gene expression in the two groups of bacteria are essentially the same; when QS bacteria are growing, they produce and release a series of molecules called autoinducers (AI) to the external environment at a low basal level. As the population increases, these molecules accumulate until they reach a certain threshold level, which leads to the activation of different sets of target genes that allow the bacteria to survive environmental
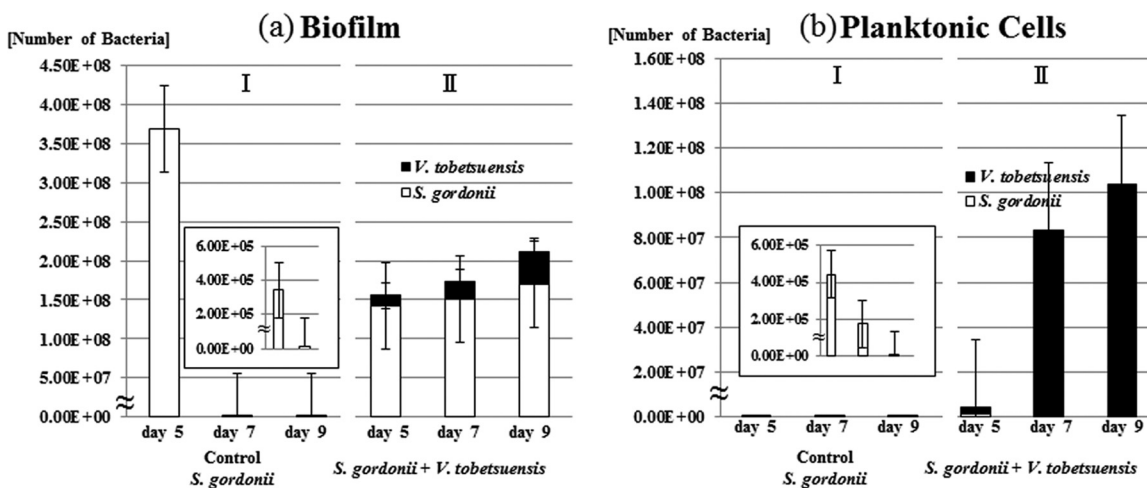

FIG 3 (a) (I) Changes in biofilm formation with *S. gordonii* as a control over time. The inset graph shows an expanded view of days 7 and 9 with *S. gordonii*. (II) Changes in biofilm formation with *S. gordonii* and *V. tobetsuensis* over time. (b) (I) Changes in planktonic cell numbers with *S. gordonii* as a control over time. The inset graph shows an expanded view of days 5, 7, and 9 with *S. gordonii*. (II) Changes in planktonic cell numbers with *S. gordonii* and *V. tobetsuensis* over time.
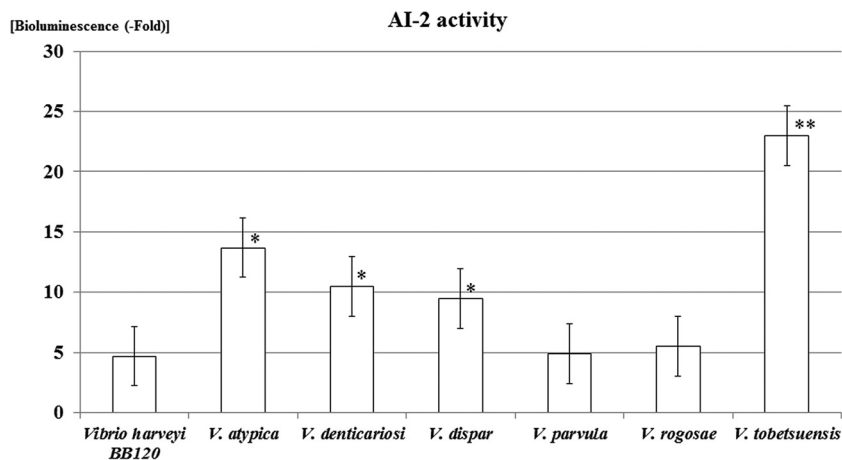
FIG 4 AI-2 activity of 6 oral *Veillonella* species. AI-2 activity = average sample value/negative value (negative control, autoinducer bioassay medium). *, $P >$ 0.05; **, $P >$ 0.01 versus control.

changes (64). The QS system is used to regulate diverse functions, such as biofilm formation (65), virulence adaptation (66), the production of antimicrobial substances (67), motility (68), sporulation (69), etc.

A number of chemically distinct families of QS molecules have been identified. The most intensively investigated family is N-acylhomoserine lactone as an autoinducer (AI-1) in Gram-negative bacteria and peptide autoinducers in Gram-positive bacteria (70).

A second type of QS system found in a wide variety of bacteria, including both Gram-negative and Gram-positive species, is termed the *luxS* or AI-2 system, and the structure of AI-2 was reported to be a furanosyl-borate diester (71). The third type of autoinducer is cholerae autoinducer 1 (CAI-1), produced by several *Vibrio* species; it has been identified and characterized as (S)-3-hydroxytridecan-4-one (72).

In the case of oral *Streptococcus* or other species, it has been reported in many studies that *luxS* or the AI-2 system is used in biofilm formation and as a virulence factor. For example, the *luxS*-based QS system affects biofilm formation in *S. anginosus*, *S. gordonii*, and *S. mutans* (73–76). In addition, in the case of *S. gordonii*, AI-2-like signaling produced by *S. gordonii* regulates aspects of carbohydrate metabolism in the organism. Furthermore, *luxS*-dependent intercellular communication is essential for biofilm formation between nongrowing cells of *S. gordonii* and *Porphyromonas gingivalis*, which is known to be a periodontal pathogenic bacterium (77).

Moreover, the mutation of *luxS* from *S. pyogenes*, which belongs to group A streptococcus, a major human pathogen that causes a wide array of diseases, affects growth and virulence factor expression in *S. pyogenes* (78). Marouni and Sela (79) also reported that *luxS* activity in *S. pyogenes* plays an important role in the expression of virulence factors associated with epithelial cell internalization. Stroeher et al. (80) reported that mutation of *luxS* in *Streptococcus pneumoniae*, which is a pathogenic human bacterium, affects its virulence in a mouse model. They were the first to investigate the direct role for *luxS* (and, by extension, AI-2).

However, the autoinducers produced by *Veillonella* species have been previously reported in a small number of studies. Frias et al. (81) demonstrated that periodontal pathogens, which, in

their report, included *V. parvula*, produced QS signaling molecules such as AI-1 and AI-2 that were detected in previously reported *Vibrio* assays (82). However, until now, their roles and functions in QS systems had not been clarified. Moreover, the QS system between oral *Streptococcus* species and *Veillonella* species has not been investigated.

When it was shown in our previous study that the greatest amount of biofilm among the 24 combinations was formed in the combination of *S. gordonii* with *V. tobetsuensis*, we hypothesized that some molecular factors, such as AI, produced by *V. tobetsuensis* might stimulate the formation of the biofilm with *S. gordonii* based on the QS system. Therefore, we focused on the QS system of *V. tobetsuensis* and tried to detect AI-1 and AI-2 in our current study. In particular, AI-2 as a strong signaling molecule was detected by the *Vibrio* assay (82) in the culture supernatants of *V. tobetsuensis*. In particular, AI-2 from *V. tobetsuensis* showed the highest activity among 6 oral *Veillonella* species in 5-day culture supernatants (Fig. 4). This result indicated that these AIs (mainly AI-2) produced by *V. tobetsuensis* may facilitate biofilm formation of *S. gordonii*.

In this article, we have reviewed studies of oral *Veillonella* and *Streptococcus* spp., which contribute to the early stages of oral biofilm formation. However, little is known about the interactions between *Veillonella* and *Streptococcus* spp., including the QS system with AIs. Although further studies are expected, we propose in conclusion that AI-2 produced from *V. tobetsuensis* may be one of the keys to revealing the mechanism of oral biofilm formation with *Streptococcus* and *Veillonella* spp.

FUTURE DIRECTIONS

It is very important to investigate the QS system of human-pathogenic bacteria as a means to prevent and treat many infectious diseases. However, dental plaque is an oral biofilm that consists of many kinds of oral bacterial species that are normal inhabitants of that niche. According to the results of our current study, biofilm formation was greatest in the combination of *S. gordonii* with *V. tobetsuensis*. Furthermore, AI-2 from *V. tobetsuensis* showed the highest activity among 6 oral *Veillonella* species. These results suggest that AI-2 from *V. tobetsuensis* may positively contribute to the biofilm

of *S. gordonii* and thus to the early stages of oral biofilm development.

In the near future, the QS system formed by autoinducers from not only *V. tobetsuensis* but also *S. gordonii* will be analyzed to clarify the mechanism of biofilm formation by these species. Furthermore, to study interactions between *S. gordonii* and *V. tobetsuensis*, including the QS system, it will be helpful to understand the unique physiology and ecology of both *Streptococcus* and *Veillonella* species. Consequently, this will lead to the development of novel methods for the prevention of oral infections, such as dental caries and periodontitis, caused by oral biofilm in early stages of development.

ACKNOWLEDGMENTS

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